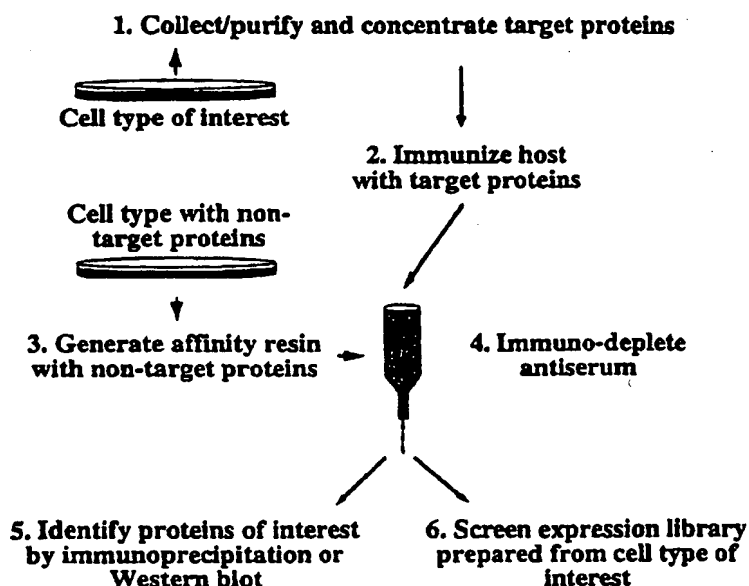




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : G01N 33/68, 33/569, 33/574	A1	(11) International Publication Number: <b>WO 98/30910</b> (43) International Publication Date: 16 July 1998 (16.07.98)
<p>(21) International Application Number: PCT/US98/00588</p> <p>(22) International Filing Date: 7 January 1998 (07.01.98)</p> <p>(30) Priority Data: 60/035,371 9 January 1997 (09.01.97) US</p> <p>(71) Applicant (for all designated States except US): WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): BICKEL, Perry, E. [US/US]; 152 Robbins Road, Watertown, MA 02172 (US). SCHERER, Philipp, E. [CH/US]; 182 Caterson Terrace, Hartsdale, NY 10530 (US). LODISH, Harvey, F. [US/US]; 195 Fisher Avenue, Brookline, MA 02146 (US).</p> <p>(74) Agents: GRANAHAAN, Patricia et al.; Hamilton, Brook, Smith &amp; Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	

(54) Title: SUBTRACTIVE ANTIBODY SCREENING (SAS) AND USES THEREFOR



## (57) Abstract

The present invention provides a method to identify and isolate proteins and genes encoding the proteins expressed in a particular cell type of interest or subcellular fraction of a cell type of interest that are not expressed in at least one other cell type. The invention provides subtracted or immunodepleted antiserum. The immunodepleted antiserum is raised against a particular cell type of interest or subcellular fraction of a particular cell type of interest, and depleted of antibodies that bind antigens from at least one other cell type or subcellular fraction of at least one other cell type. The immunodepleted antiserum is used to screen an expression library generated from the particular cell type of interest.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

-1-

# SUBTRACTIVE ANTIBODY SCREENING (SAS) AND USES THEREFOR

## FUNDING

Work described herein was funded in whole or in part by Grant Numbers DK 47618 and DK 02219 from the National  
5 Institutes of Health. Accordingly, the United States Government has certain rights in the invention.

## RELATED APPLICATION

This application claims the benefit of U.S. Serial number 60/035,371, entitled Subtractive Antibody Screening  
10 (SAS) and Uses Therefor, by Perry E. Bickel, Philipp E. Scherer and Harvey F. Lodish, filed January 9, 1997. The

-2-

teachings of U.S. Serial number 60/035,371 are incorporated expressly herein by reference.

#### BACKGROUND OF THE INVENTION

The ability to identify and clone genes expressed in  
5 specific tissues and disease states has great value for  
drug discovery and development of diagnostic tools. To  
date, strategies that address this critical goal are  
limited. Typical cDNA expression libraries and screening  
methods are limited because they are dependent upon the  
10 different levels of messenger RNA encoding specific  
proteins. Unfortunately, the genes of interest are not  
always represented by high levels of corresponding mRNA.

Proteins of interest are often those expressed in a  
tissue specific or disease state specific manner. Attempts  
15 to improve the likelihood of obtaining differentially  
expressed genes involve generation of subtractive cDNA  
libraries, whereby the cDNAs common between two or more  
cell types are removed from the library. This method is  
also limited by the relative abundance of specific mRNA  
20 molecules encoding proteins of interest. Messenger RNA  
expression may not adequately represent the proteins of  
greatest interest.

Furthermore, the preceding methods do not promote the  
cloning of genes encoding proteins localized in specific  
25 subcellular compartments of a cell (e.g. nuclear,  
cytoplasmic, organellar, plasma membrane, intracellular  
membrane or secretory proteins). In particular, plasma  
membrane and secretory proteins are of great interest as  
potential drug targets.

-3-

Specialized cDNA libraries have been constructed (U.S. Patent 5,536,637) that promote the cloning of genes encoding secretory proteins that possess a signal sequence, whereby the cDNA is ligated to DNA encoding a non-secreted yeast invertase. Those constructs which subsequently allow invertase auxotrophic yeast to proliferate on sucrose medium are selected. Thus, genes possessing a signal sequence may be cloned. However, not all genes of interest possess a signal sequence, even of those genes encoding plasma membrane proteins. For example, type II plasma membrane proteins (whose c-terminus is extracellular) do not possess a typical signal sequence.

Further, the process of cloning and characterizing a novel gene and encoded protein is a time consuming and labor intensive process. Once it is determined that a sequence is novel, laborious characterization is necessary to determine whether the gene and encoded protein are of interest or are potential drug candidates based on expression pattern and response to stimuli. In order to accomplish these goals, antibodies specific for the novel protein are useful to characterize, purify, monitor and manipulate the novel protein. The methods described thus far do not facilitate such processes in any way.

#### SUMMARY OF THE INVENTION

Described herein is a method of identifying and isolating proteins of interest that are expressed in a particular cell type or tissue type, and not expressed in at least one other cell type or tissue type (the subtractive cell type or tissue type). Also described herein is a method of identifying and cloning genes that

-4-

encode the proteins of interest. The present method therefore allows the identification and cloning of genes expressed in specific cell types or tissues. Genes identified by the present method, which are expressed in a particular cell type or types and not in the subtractive cell type, proteins of interest and antisera specific for said proteins of interest are also the subject of the present invention.

In the present method, cDNA expression libraries are screened, using immunodepleted antisera which have been produced, as described herein, in such a manner that they contain antibodies which recognize (bind) antigens present in a particular cell type or types but not in at least one other cell type. This method is referred to herein as subtractive antibody screening (SAS). Further, the present invention provides a method to remove from antisera antibodies that react with antigens found in cells other than the cell type of interest, as well as antibodies that react with antigens of previously identified proteins.

The method of identifying a protein expressed in a cell type of interest and not expressed in at least one cell type other than the cell type of interest includes combining a suitable cDNA expression library and immunodepleted serum. The cDNA library can be from the cell type of interest or a different suitable cell type.

Proteins expressed by the cDNA expression library are bound by antibodies in the immunodepleted serum to form complexes. The complexes of proteins expressed by the cDNA expression library and antibodies in the immunodepleted serum are detected. The presence of complexes indicates the expression of a protein in the cell type of interest,

-5-

and the lack of expression in a cell type other than the cell type of interest. Further, the presence of complexes indicates the presence of the gene or gene fragment encoding said protein.

5       The method of generating immunodepleted serum includes combining: 1) antiserum produced by immunizing a suitable host with proteins derived from the cell type of interest and 2) proteins obtained from at least one cell type other than the cell type of interest, under conditions  
10 appropriate for binding of antibodies in the antiserum to the antigens, producing antigen-bound antibodies, referred to as antibody-antigen complexes. The antibody-antigen complexes can be separated from the resulting immuno-  
15 depleted antiserum, but it is not necessary that this be done. The immunodepleted serum, therefore, is enriched in antibodies that recognize proteins expressed in the cell or tissue of interest and that are not expressed in at least one other cell or tissue type.

      The cell type of interest can be from any type of  
20 tissue, for example, normal tissue, metastatic malignant tissue, non-metastatic malignant tissue, cultured cells, or immortalized (transformed) cultured cells. The cell type of interest can be differentiated. The cell type of interest can be adipocytes, fibroblasts, C2C12 osteoblasts,  
25 C2C12 myotubes, neurocytes such as PC12 cells or HNT cells (Stratagene). The cell type of interest can be blood cells. The cell type of interest can be progenitors of the cell types listed above.

      In the embodiments of the method, the cell type of  
30 interest and the cell type other than the cell type of interest (subtractive cell) can be from the same or

-6-

different tissue or cell source. The cell type of interest can be stimulated or not, and the subtractive cell can be non-stimulated or stimulated, respectively. The cell type of interest can be normal and the subtractive cell can be transformed, malignant or metastatic. Conversely, the cell type of interest can be transformed, malignant or metastatic malignant and the subtractive cell can be normal. In addition, the cell type of interest can be transformed, malignant or metastatic and the subtractive cell non-transformed, non-malignant or non-metastatic, respectively. The cell type of interest and the subtractive cell type can be reversed (e.g., the cell type of interest can be non-transformed cells and the subtractive cell can be transformed cells). Further, the cell type of interest can be adipocytes and the subtractive cell can be fibroblasts.

Proteins used to immunize the host can be derived from subcellular fractions of a cell type of interest, such as secreted, plasma membrane, cytoplasmic, organellar, subcellular membrane or nuclear proteins. Further, the proteins used to immunize the host can be derived from subfractions thereof, such as carbohydrate-containing plasma membrane proteins.

The proteins obtained from at least one cell type other than the cell type of interest can be modified to allow separation of antibody-antigen complexes from the antiserum by coupling the antigens to a suitable solid support, such as Sepharose, agarose, acrylamide, nitrocellulose, glass, plastic or silicone, or by chemically adding a suitable functional group, such as biotin or the steroid hapten digoxigenin, to the proteins.



-7-

A cDNA encoding a protein expressed in a cell type of interest is identified by combining a cDNA expression library and the immunodepleted serum, whereby proteins expressed by the cDNA expression library bind antibodies in the immunodepleted serum to form complexes of a protein expressed by the cDNA expression library and an antibody in the immunodepleted serum. The complexes of proteins expressed by the cDNA expression library and antibody from the cDNA expression library are detected. The cDNA library may be generated from any suitable cell type, including the cell type of interest.

A gene or fragment thereof encoding the detected protein expressed in a cell type of interest is isolated from the cDNA expression library by selecting a member of the cDNA expression library expressing the detected protein and isolating the cDNA encoding the protein.

The cell type of interest can be derived from normal tissue, metastatic malignant tissue, non-metastatic malignant tissue, cultured cells or immortalized (transformed) cultured cells. The cell type of interest can be stimulated, or unstimulated. Stimulation can include chemical addition or physiological change. Malignant or malignant-metastatic transformation and immortalization can result from chemical treatment or genetic transformation or can occur spontaneously.

As described herein, adipocyte specific proteins have been identified and cloned. The cloning of leptin has demonstrated the relevance of the adipocyte to the prevalent diseases of diabetes mellitus and obesity. This technology also can be applied to other, diverse biomedical systems that have relevance to human disease and,

-8-

therefore, can provide targets for drug discovery. The present methods can be applied to any system in which expressed proteins can be isolated before and after a relevant stimulus.

5           The present method has several advantages. The present method promotes the cloning of genes encoding proteins expressed in specific cell types or specific subcellular fractions of said cell types. More specifically, the present method promotes the cloning of  
10 genes encoding proteins from subcellular fractions (such as plasma membrane proteins and secretory proteins) that are of great importance in drug targeting and pharmaceutical development. Further, the present method facilitates the cloning of these genes whether or not a signal sequence is  
15 present.

Another advantage of the present method is that at the time of cloning a gene of interest, antibodies directed against the encoded protein exist in the immunodepleted antiserum of the present invention that was used for the  
20 cloning. The specific antibody can be affinity-purified from the immunodepleted sera and used for initial characterization of novel proteins. Therefore, as soon as a clone is identified, the expression of its protein can be examined for desired characteristics such as response to  
25 hormone treatment. Thus, the entire process of gene discovery and protein characterization is streamlined and accelerated.

#### DESCRIPTION OF THE DRAWING

The Figure is a schematic diagram of the method of the  
30 present invention.

## DETAILED DESCRIPTION OF THE INVENTION

The novel method described herein is useful to systematically identify proteins that are expressed in specific tissues or cell types and are not expressed in other tissue or cell types and, optionally, to clone genes encoding these proteins.

Many proteins that are drug targets are either secreted by cells (hormones, cytokines, growth factors, enzymes, extracellular matrix (ECM), adhesion molecules) or expressed on the cell surface (receptors, transporters, channels, ECM receptors, adhesion molecule receptors). The present invention is particularly useful for identifying these subsets of cellular proteins. As described herein, the subtractive antibody screening method has been used to clone genes encoding cell-type specific proteins. In one embodiment, it has been used to clone genes encoding secreted proteins (e.g., a protein similar to Type VI collagen) and cell surface proteins whose expression is induced during adipogenesis. The discovery of a collagen whose expression is induced during adipogenesis has important implications for adipocyte differentiation and provides a drug target to be assessed for the treatment of obesity.

The present method is useful to identify expressed proteins from a particular (target) tissue or cell type. The method is useful to identify proteins that are expressed in a target tissue or cell type and not expressed in other (subtractive) tissue or cell type(s). The present method is useful to identify and, optionally, clone genes encoding proteins from a particular tissue or cell type of interest before and after a relevant stimulus. Furthermore, the present methods can be applied to expressed proteins from a particular tissue or cell type of

-10-

interest relative to other tissue(s) or other cell type(s) without treatment or stimulation.

The cells of interest (either stimulated or not stimulated) are referred to herein as the "target tissue(s) or target cell(s)". The term "subtractive tissue(s)" or "subtractive cell(s)" refers to cell types or tissues other than the cell type of interest. In the case in which target cells are stimulated cells of a particular type, subtractive cells can be nonstimulated cells of the same type, nonstimulated cells of another type or types or stimulated cells of another cell type. Furthermore, in the case in which target cells are nonstimulated cells of a particular type, subtractive cells can be stimulated cells of the same type, stimulated cells of another type or types or nonstimulated cells of another cell type. Similarly, in those embodiments in which the cell type of interest is a transformed cell, a malignant cell or a metastatic cell, the cell type other than the cell type of interest can be, for example, respectively, the corresponding nontransformed cell, nonmalignant cell or nonmetastatic cell. Alternatively, the cell type other than the cell type of interest can be cells of another type or types (not the same cell type as the cell type of interest).

The method, referred to as subtractive antibody screening (SAS), is useful to identify and clone genes and identify and, optionally, isolate, the encoded proteins from any type of target cell, including procaryotic (e.g. bacterial) and eucaryotic (e.g., fungal, insect, plant, vertebrate, mammalian, human) cells for which expressed proteins can be isolated. Human tissue and cell types, including, but not limited to, endothelial cells, epithelial cells, hepatocytes, fibroblasts, nervous tissue, blood cells, muscle cells, cells of the immune system (e.g. macrophage, mast cells, T cells, B cells), transformed

-11-

cells (i.e. immortalized cells) and malignant transformed cells (i.e. immortalized and non-contact inhibited cells) and metastatic malignant cells can be screened by the method described herein to identify proteins and the genes encoding the proteins.

The target cells can be derived directly from an organism, derived from an organism and then passaged in culture (primary culture) or derived from an immortalized cell line. The tissue of interest, which includes the target cells, may comprise one cell type, such as in nervous tissue, or multiple cell types, such as in connective tissue. The tissue of interest can be employed immediately after isolation from an organism, after a period of incubation or after being maintained by perfusion or other means of preservation.

In one embodiment, the target cells and subtractive cell counterparts may be, respectively, stimulated and nonstimulated versions of the same cell type or tissue of interest. In another embodiment, the target cell and subtractive cell counterparts may be two different cell types or tissues, either stimulated or not.

The stimulus to which target cells are subjected can be, for example, a physiological change (e.g., altered temperature, osmolarity or nutritional supplement), shear stress, ischemia, hormone treatment, cellular transformation, incubation for a period of time (in the case of tissue samples or primary cell culture) or chemical treatment. Further, the stimulus can be a differentiation protocol involving one or more of the previously described stimuli.

Subtractive antibody screening is carried out by immunoscreening a cDNA expression library from a suitable cell type with polyclonal antisera. As used herein, the term "cDNA expression library from a suitable cell type"

-12-

refers to a collection of genes expressed in the cell type. The cDNA expression library is a library that represents the transcribed genes of a cell, at the same relative abundance as that found in the cell. The cDNA expression library is prepared from a suitable cell type using methods known to those of skill in the art (see, e.g. pages 7.1-7.35, 8.1-81 and 12.1-12.44, Molecular Cloning, a Laboratory Manual Second Edition, J. Sambrook et al., (Eds.); pages 4.0.1-5.11.2, Current Protocols in Molecular Biology, F. Ausubel et al., (Eds.)) and introduced into the appropriate host cell. In one embodiment, the cDNA expression library is prepared from the target cells in which protein expression is to be assessed. The expression library is prepared from the mRNA of the cells. The mRNA is inserted as a cDNA copy into any vector that allows expression of the cDNA insert in a host cell (expression host). Many suitable expression vectors are known to those of skill in the art, as are suitable expression hosts (such as bacteria, yeast, insect cells and vertebrate cells, including mammalian cell culture) for the particular expression vector. In another embodiment, the cDNA library can be a subtractive cDNA library. Methods for generating subtractive cDNA libraries are well known in the art.

Polyclonal antiserum is prepared against proteins of the target cells according to known methods (see, e.g., pages 53-137, Antibodies, a Laboratory Manual, E. Harlow and D. Lane, (Eds.)) and is depleted of antibodies that react with proteins expressed in subtractive cells.

The proteins used to deplete the polyclonal antiserum of antibodies that react with proteins expressed in subtractive cells are referred to as immunodepletion antigens and can be derived from progenitors or derivatives of the target cells (e.g., before treatment or stimulation or after) or can be derived from a separate cell sample

-13-

which has not been treated or stimulated. The immunodepletion antigens isolated from the subtractive cells can be modified to facilitate isolation of the antigens and bound antibodies away from solution. The antigens can be immobilized onto a solid support such as a resin (e.g. Sepharose, agarose or acrylamide) or other solid support, such as nitrocellulose, glass, plastic or silicone. Alternatively, the immunodepletion antigens can be chemically modified to facilitate their removal from solution. For example, immunodepletion antigens can be chemically modified with biotin to facilitate isolation using immobilized avidin or streptavidin. Immunodepletion antigens can also be chemically modified with the steroid hapten digoxigenin to facilitate isolation using immobilized anti-digoxigenin antibodies.

Immunodepleted serum, which is serum from which antibodies that bind proteins expressed in at least one cell type other than the target cell type have been removed, is prepared by 1) combining the polyclonal serum, described above, and the immunodepletion antigens; and 2) maintaining the resulting combination under conditions appropriate for antigen-antibody binding to occur, resulting in antigen-bound antibodies and thus, producing immunodepleted serum. In another embodiment, the antigen-bound antibodies are removed from the polyclonal serum.

Immunoscreening is carried out under conditions appropriate for binding of antibodies with antigens(see, e.g. pages 12.1-12.44, Molecular Cloning, a Laboratory Manual Second Edition, J. Sambrook et al., (Eds.); and pages 6.0.1-6.12.12, Current Protocols in Molecular Biology, F. Ausubel et al., (Eds.)). Expression hosts expressing the cDNA library are induced with conditions that permit expression of the cDNA. The expression hosts are combined with immunodepleted serum under conditions

-14-

appropriate for specific binding of antibodies to protein expressed by the cDNA to occur, wherein antibodies that bind proteins of interest but do not bind proteins from a cell type other than the cell type of interest are specific antibodies, with the result that complexes of a protein expressed by the cDNA expression library and an antibody in the immunodepleted serum are generated. Complexes of antibodies in the immunodepleted serum bound to proteins in the cDNA expression library are detected. Presence of complexes is indicative of the presence of a protein expressed in the cell type of interest. Expression host cell(s) harboring the cDNA expressing an antibody-bound protein is/are identified. In one embodiment, the expression hosts are immobilized on a solid support and then lysed or permeabilized, if necessary, to allow antibody binding. In another embodiment, the expression host cell(s) are permeabilized, if necessary, and combined or contacted with immunodepleted serum, under conditions which permit antibody antigen binding. The antibody-bound expression hosts can be isolated by fluorescence-activated cell sorting or by binding to Protein A-coated beads followed by isolation of the beads together with antibody bound expression hosts.

The nucleic acid (DNA, RNA) encoding the protein can be isolated using methods well known in the art from the expression host(s) identified as described above. The isolated nucleic acid (DNA, RNA) can also be amplified and characterized using methods well known in the art. Further, the isolated nucleic acid can be used as a probe to isolate full length cDNA encoding the protein of interest (if it is not already a full length clone), or can be used as a probe to isolate related sequences, such as those encoding homologues or family members of the protein of interest.



In one embodiment, proteins of a particular subcellular fraction of target cells are used to generate polyclonal antisera as described above. Proteins from the corresponding subcellular fraction of the subtractive cells  
5 are immobilized as described above and used to immunodeplete the polyclonal antiserum generated against antigens from the target cells. Proteins from lysates of subtractive cells can also be employed as immunodepletion antigens. In one embodiment, post nuclear supernatants  
10 (whole cell lysates with nuclei removed) are used to immunodeplete the polyclonal antiserum. The polyclonal antiserum can be further immunodepleted against known antigenic proteins such as hsp60 and hsp70.

The resulting immunodepleted antiserum is used to  
15 screen an expression library (e.g., a lambda phage expression library) prepared from a suitable cell type or from target cells of interest or target cells treated in the same manner as target cells used to raise the polyclonal serum. Proteins bound by the antibodies of the  
20 immunodepleted antiserum are proteins expressed in the target cells but not in the subtractive cells used to generate the immunodepletion antigens. As a result, proteins expressed in the target cells or subcellular fraction of target cells and not expressed in subtractive  
25 cells are identified. Antibody-bound antigens and DNA encoding the bound antigens are identified and isolated using known methods. The isolated DNA can be sequenced and analyzed.

The polyclonal antiserum can be further immunodepleted  
30 against proteins identified using this method, to prevent redundant identification in subsequent immunoscreening procedures.

To simplify the description of the following embodiments, the condition where the target cells are

-16-

stimulated and the subtractive cells are the same type of cells as the target cells, but are not stimulated is described. It is understood that in a different embodiment, the target cells can be unstimulated and the subtractive cells can be the same type of cells as the target cells, but are stimulated. Further, in another embodiment the target cells can be one type of cell and the subtractive a different type of cell, both types stimulated or not.

10 In one embodiment, genes encoding proteins from a subcellular fraction of the target cells are cloned. For example, cytoplasmic proteins can be identified and cloned by the present method. In this embodiment, subtractive cells are lysed and fractionated according to known methods  
15 to isolate cytosol away from membranes, organelles and nuclei. Proteins released from the cells (proteins in the cytosol) are immobilized onto a solid support as described above. Proteins from lysates of subtractive cells can also be employed as immunodepletion antigens (as described  
20 above). Known highly antigenic proteins such as hsp60 and hsp70 can also be employed as immunodepletion agents to reduce the likelihood that such non-target proteins will not be detected in the screening process.

Target cells are lysed in the same manner as described  
25 above and the isolated cytoplasmic proteins are used to generate polyclonal antiserum according to known methods. The resulting antiserum is then immunodepleted of antibodies that bind antigens isolated from the subtractive cells by reacting the polyclonal serum with the proteins  
30 immobilized as described above. The resulting antiserum is then used to immunoscreen a suitable cDNA expression library. Antibodies in the immunodepleted serum recognize, bind and form complexes with proteins expressed by the cDNA expression library. The antigen-antibody complexes are

-17-

detected, and the protein and polynucleotide encoding the protein are identified and optionally isolated.

Nuclear proteins can also be identified and cloned by the present method. For example, nuclei can be isolated  
5 from subtractive cells and nuclear extracts prepared according to known methods. Proteins in the resulting nuclear extracts are immobilized onto a surface as described above. Proteins from whole cell lysates of subtractive cells can also be employed as immunodepletion  
10 antigens (as described above). Known antigenic proteins such as hsp60 and hsp70 can also be employed as immunodepletion agents.

Nuclei from target cells are isolated and nuclear extract prepared according to standard methods known in the  
15 art. Polyclonal antiserum is produced against the preparation of nuclear extract from the target cells. The resulting antiserum is immunodepleted of antibodies that bind antigens isolated from the subtractive cells. The resulting immunodepleted antiserum is then used to  
20 immunoscreen a suitable cDNA expression library. Antibodies in the immunodepleted serum recognize, bind and form complexes with proteins expressed by the cDNA expression library. The antigen-antibody complexes are detected, and the protein and polynucleotide encoding the  
25 protein are identified and optionally isolated.

In another embodiment, subtractive antibody screening can be used to identify and clone secreted proteins and genes encoding these proteins from the target cells. Polyclonal antisera are raised against the total complement  
30 of secretory proteins from the target cells. This antiserum is then immunodepleted of binding activity against secreted proteins from the subtractive cells, by reacting the polyclonal serum with the immobilized secretory proteins of the subtractive cells. Proteins from

-18-

whole lysates of subtractive cells can also be employed as immunodepletion antigens (as described above). Known antigenic proteins such as hsp60 and hsp70 can also be employed as immunodepletion agents. The resulting

5 immunodepleted antiserum is used to immunoscreen a suitable cDNA expression library. Antibodies in the immunodepleted serum recognize, bind and form complexes with proteins expressed by the cDNA expression library. The antigen-antibody complexes are detected, and the protein and

10 polynucleotide encoding the protein are identified and optionally isolated.

As described in the Examples, the present method has been carried out to identify proteins expressed in adipocytes (target cells) and not expressed in fibroblasts

15 (subtractive cells). Specifically, 3T3-L1 fibroblasts were used. As described in Example 1, 3T3-L1 fibroblasts were induced to differentiate into adipocytes over eight days. Two-dimensional gel electrophoresis was used to demonstrate that at least thirty proteins are secreted by adipocytes

20 that are not secreted by fibroblasts. Polyclonal antisera were raised against the total complement of secretory proteins from adipocytes. To do this, serum-free conditioned media from 3T3-L1 adipocytes were collected and concentrated. This material was used to immunize rabbits,

25 and the resulting antisera were collected. The total complement of secretory proteins from fibroblasts and protein from lysed fibroblasts were collected, concentrated and used to generate the immunodepletion agent as described above and in Example 2. The polyclonal antisera were

30 systematically immunodepleted of antibodies that react with the immobilized fibroblast proteins described above. Known antigenic proteins such as hsp60 and hsp70 were also employed as immunodepletion agents. Antisera subjected to several such depletion steps were highly enriched in

-19-

antibodies that recognize adipocyte-specific secretory proteins.

As described in Example 4, the immunodepleted antiserum recognizes several proteins whose expression and/or secretion is modulated by chronic insulin treatment. This antiserum also recognizes at least eight circulating proteins from mouse serum. The immunodepleted antiserum was used to immunoscreen a lambda phage expression library prepared from 3T3-L1 adipocytes. This resulted in the identification of a number of genes encoding previously identified proteins, as well as at least five novel genes. Blast search at the National Center for Biotechnology and Information and Northern blot analysis was used to confirm the tissue specificity of the isolated clones. More than half of the positive clones identified with the subtractive antibody screening method described herein represented known secretory proteins. Some proteins identified are known to be secreted by adipocytes (e.g., fibronectin, complement factor C3) and some not previously described as being adipocyte secreted proteins (e.g., osteonectin and alpha-1 acid glycoprotein). As described in Example 7, Northern blot analysis of mouse tissues showed that mouse adipose tissue has higher levels of osteonectin messenger RNA per microgram of RNA than any other tissue examined. Thus, the unexpected cloning of osteonectin with the subtractive antibody screening method reflects a true adipose tissue phenomenon, and not merely a cell culture artifact.

As described in Example 7, one of the novel genes cloned demonstrates homology to the alpha 3 subunit of human Type VI collagen ( $\alpha 3(\text{VI})$  collagen). The expression of the gene is elevated in adipocytes, as determined by Northern blot. Discovery of a collagen that is induced during adipocyte differentiation is significant because of

-20-

the role that the ECM plays in mesenchymal cell differentiation. Adipocyte precursors share a common lineage with chondrocyte, osteoblast, and smooth muscle cell precursors. In the case of osteoblasts, cytokines  
5 such as TGF $\beta$  and bone morphogenic proteins are sequestered and concentrated by the ECM, and these proteins influence osteoblast differentiation. Similarly, the ECM that surrounds adipocytes may trap soluble factors that influence adipocyte differentiation and/or function. The  
10 ECM also forms the physical bed in which the adipocytes lie. Thus, the collagen identified and cloned by the present method is a promising target for modulating adipocyte differentiation and function.

In another embodiment, cell surface proteins can be  
15 identified and cloned by the present method. For example, intact subtractive cells are surface labeled (e.g., by addition of a member of a binding pair, such as biotin) before stimulation and the resulting labeled (e.g., biotinylated) proteins are immobilized on a solid support  
20 (e.g., a resin) that has attached thereto the second member of the binding pair (e.g., streptavidin). Intact target cells are stimulated and then surface labeled (e.g., by addition of a member of a binding pair, such as biotin) and the resulting labeled (e.g., biotinylated) proteins are  
25 purified over a solid support (e.g., a resin) which has attached thereto the second member of the binding pair (e.g., streptavidin). Proteins bound to the solid support as a result of binding of biotin with streptavidin are surface proteins of the stimulated target cells. In one  
30 embodiment, the solid support can be washed to remove proteins that associate indirectly with the plasma membrane (e.g. via a biotinylated protein). Such conditions include high salt concentration, high pH or the presence of an ionizing detergent, such as SDS. The labeled proteins are

-21-

released and polyclonal antiserum is generated against the eluted material. This antiserum is then immunodepleted of activity against proteins from the subtractive cells by reacting the polyclonal serum with the biotinylated, 5 immobilized proteins described above. Further, the polyclonal antiserum can be immunodepleted against surface proteins of intact live cells. Known antigenic proteins such as hsp60 and hsp70 can also be employed as immunodepletion agents. The resulting antiserum is then 10 used to immunoscreen a suitable cDNA expression library. Antibodies in the immunodepleted serum recognize, bind and form complexes with proteins expressed by the cDNA expression library. The antigen-antibody complexes are detected, and the protein and nucleotide encoding the 15 protein identified and optionally isolated.

In a particular embodiment of a method of identifying and cloning the genes for surface proteins, intact adipocytes (target cells) were biotinylated with sulfo-NHS-SS-biotin (Pierce). The resulting biotinylated proteins 20 were purified over a solid support (e.g., a resin) that had streptavidin attached thereto. Proteins bound to the solid support as a result of binding of biotin with streptavidin were adipocyte proteins. The surface-labeled proteins were released from the solid support using reducing conditions 25 to break the disulfide bond in the NHS-SS-biotin (according to the manufacturer's instructions) and used to immunize rabbits. The resulting antiserum contained a high titer of antibodies against adipocyte surface proteins, as determined by immunofluorescence. This antiserum was then 30 depleted of activity against fibroblast (subtractive cells) surface proteins, post nuclear supernatant proteins, and against hsp60 and hsp70 as described above for secreted proteins. Further, the polyclonal antiserum was immunodepleted against surface proteins of intact live

-22-

fibroblasts. Intact, live fibroblasts were washed with ice cold phosphate buffered saline to remove serum proteins and incubated with the immunodepleted polyclonal antisera at 4° C for 1-12 hours. The antiserum was harvested away from the cells and used for cloning. Several novel cDNA clones were identified, one of which has been fully characterized and shown to be adipocyte specific.

In another embodiment, intact adipocytes can be employed to affinity purify adipocyte-specific antibodies. Immunodepleted serum is incubated at 4°C with washed, allowing binding antibodies with adipocyte surface proteins. Adipocytes are washed to remove unbound antibodies. Bound antibodies are eluted using methods well known in the art. In one embodiment, antibodies are eluted from the cells with 100 mM glycine at pH 2.5. After elution, the pH is neutralized with 1 M Tris, pH 8.0. The resulting affinity purified antibodies can be used to immunoscreen a suitable cDNA library.

In other embodiments, genes encoding proteins from subcellular fractions such as organellar fractions or subcellular membrane fractions other than the plasma membrane can be cloned. These subcellular fractions can be isolated using methods well known in the art. Further, genes encoding proteins from subfractions thereof, such as carbohydrate-containing plasma membrane proteins can be cloned.

In one embodiment, the present method is useful to identify proteins and clone the genes encoding the proteins expressed at different stages of development. For example, genes encoding proteins expressed at specific stages or in specific regions of a developing embryo are systematically identified and cloned from appropriately stimulated embryonic stem cells or from fertilized eggs. The stimulated stem cells or the fertilized eggs can be



-23-

maintained *in vitro* or *in vivo*. The fertilized eggs can be from any organism suitable as a source for eggs, such as spistula clam, drosophila, sea urchin, frog, chicken or mouse.

- 5           In another embodiment, genes encoding proteins expressed at specific stages of differentiation in a wide variety of cell types can be identified and cloned. For example, genes encoding proteins expressed at specific stages of blood cell differentiation, neuronal cell
- 10 differentiation, osteoblast differentiation, myotube differentiation or epithelial cell differentiation can be cloned from appropriately treated progenitor cells, using standard methods known in the art. Blood cells such as mast cells, B cells, T cells, macrophages, erythroblasts
- 15 and megakaryocytes can be generated from appropriately stimulated hematopoietic stem cells. Neuronal cells can be generated from appropriately stimulated neuronal cells such as PC12 cells or HNT cells (Stratagene). Osteoblasts can be generated from appropriately stimulated C2C12 cells.
- 20 Myotubes can be generated from appropriately stimulated C2C12 cells.

The present method is also useful to systematically identify and clone genes encoding proteins that are expressed in a wide variety of abnormally proliferating

25 cells, including malignant, malignant metastatic and immortalized cells. These abnormally proliferating cells can be obtained from sources in which they occur in nature (e.g., tumors) or be induced by chemical treatment or oncogene transfection of normal cells to generate

30 transformed or malignant cells or of transformed cells to generate malignant cells.

In one embodiment, genes specific for stressed (but not non-stressed) tissue or primary cell culture are cloned. In a more specific embodiment, tissue or primary

-24-

cell culture is exposed to shear stress or stressed with heat, cold, osmolarity, nutrition or ischemia. In another embodiment, genes specific for tissue or cell culture treated with a specific hormone, cytokine or other biological agent (but not non-treated tissue or cell culture) are cloned. In another embodiment, genes expressed in a particular type of tissue (but not in at least one other type of tissue) are cloned. In yet another embodiment, genes specific for freshly isolated tissue (but not in corresponding primary culture) are cloned.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all cited references are hereby incorporated herein by reference.

#### EXAMPLES

Example 1            Generation of polyclonal antiserum against proteins secreted by adipocytes.

Sixty 15 centimeter diameter culture dishes of 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were differentiated according to a standard protocol (Frost, S.C., and Lane, M.D. (1985) *J. Biol Chem* 260:2646-2652). After 8 days, the cells were washed five times with Dulbecco's Modified Eagle's Medium (DMEM) to remove all traces of fetal calf serum from the culture medium. Each dish of cells was subsequently incubated in 10 ml serum-free DMEM; 30 of the plates were also supplemented with 160 nM insulin. After 8 hours, the culture media were harvested. Detached cells were removed by centrifugation (10,000 g for 20 min).

The proteins secreted by the adipocytes were concentrated with Centricon units (Amicon Inc.) using a

-25-

molecular mass cutoff of 2000 Da. The proteins from this subcellular fraction were mixed with Ribi Adjuvant (RIBI Immunochem Research Inc) according to the manufacturer's instructions and injected subcutaneously into rabbits at 4 week intervals. Each injection contained about 250  $\mu$ g of total protein as judged by Coomassie-Blue staining of a small fraction of the sample. Antiserum was collected from the immunized rabbits.

- Example 2            Generation of immunodepletion antigens
- 10            To generate immobilized antigens from the material secreted from the cells of interest for immunodepletion of the polyclonal sera of Example 1, serum free media from thirty 15 centimeter diameter culture dishes of preconfluent fibroblasts were harvested and concentrated as described above, dialyzed overnight against 100 mM Na-Carbonate Buffer, pH 8.5, containing 500 mM NaCl, and subsequently coupled to CNBr-Sepharose (Pharmacia) according to the manufacturer's instructions, thus immobilizing immunodepletion antigens of secreted proteins.
- 20            In parallel, preconfluent fibroblasts were lysed in TNET-OG (1% Triton X-100, 60 mM octyl-glucoside, 150 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 1 mM PMSF). The nuclei were removed by centrifugation and the resulting supernatant was dialyzed against carbonate buffer and
- 25            coupled to CNBr-Sepharose as described above, thus generating immobilized immunodepletion antigens of total fibroblast proteins.

It is clear that many factors can be altered such as the medium used to harvest the antigens, the detergent used to harvest the antigens, the dialysis buffer and the coupling column in order to attain the desired outcome of immobilized antigens representative of the proteins of a

-26-

tissue or cell line to be used to immunodeplete the polyclonal sera of Example 1.

**Example 3            Generation of Immunodepleted antisera for subtractive antibody screening**

5            Antiserum collected from the immunized rabbits in Example 1 was incubated overnight at 4°C with the immobilized immunodepletion antigens of secreted proteins from Example 2, to deplete the antiserum of antibodies recognizing antigens present in the pre-adipocyte  
10 fibroblasts used as the source of immunodepletion antigens. This process can be repeated several times with one or more immunodepletion resins to facilitate depletion of the antisera. Additionally, in order to deplete the serum of antibodies against intracellular proteins known to have  
15 very high antigenicity such as heat shock proteins of the hsp60 and hsp70 families, T7 gene 10/hsp60 and T7 gene 10/hsp70 fusion proteins were transferred to nitrocellulose and these membranes were used for immunodepletion.

20            **Example 4            Immunoprecipitation of <sup>35</sup>S-labeled proteins secreted by day 8 3T3-L1 adipocytes**

Two 10 centimeter diameter plates of 3T3-L1 adipocytes were incubated overnight in serum-free medium either in the absence or the presence of 1 μM insulin. The plates were then labeled for 30 minutes with 1 mCi of <sup>35</sup>S-Express  
25 protein labeling reagent, again in the presence or absence of insulin. The medium was removed and plates washed 3 times with DMEM. The plates were then incubated for an additional 3 hours with or without insulin. The media were then harvested, insoluble debris removed by centrifugation  
30 and the supernatants either analyzed directly (5% of total material) or immunoprecipitated with the immunodepleted antiserum. A distinct subset of the total mixture of

-27-

secreted proteins was immunoprecipitated by the immunodepleted serum. The level of expression of some of these proteins was affected (either repressed or induced) by chronic insulin treatment.

5    Example 5            Construction of a directional 3T3-L1  
                             adipocyte phage cDNA library

          Poly(A)<sup>+</sup> RNA (5 µg) from 3T3-L1 adipocytes at day  
          eight of differentiation was used as the template to  
          construct a λEXlox™ library according to the  
10    manufacturer's instructions (Novagen, Inc., Madison, WI).  
          Oligo(dT) was used to prime the first-strand cDNA  
          synthesis. Prior to ligation to the phage arms, the cDNA  
          was size-fractionated (≥1.5 kb) using a 5%-20% potassium  
          acetate gradient (Aruffo, A., and Seed, B. (1987) *Proc.*  
15    *Natl. Acad. Sci. U.S.A.* 84:8573-8577). The resulting  
          library represented approximately 1 X 10<sup>6</sup> independent  
          clones. The library was amplified once and used for  
          screening.

          Example 6            Expression cloning.

20           Screening of 5x10<sup>5</sup> phage particles was done according  
          to standard protocols with minor modifications as described  
          in the λEXlox™ manual (Novagen, Inc.). Nitrocellulose  
          filters were blocked in TBS-T (50 mM Tris, pH 8.0, 150 mM  
          NaCl, 0.05% Tween 20) containing 1% Bovine Serum Albumin  
25    (TBS-T/BSA). Antibody incubations were performed in TBS-  
          T/BSA overnight at 4°C. Filters were washed 4 times in  
          TBS-T, incubated with 20U of digoxigenin-coupled anti-  
          rabbit antibodies (Boehringer Inc.) in TBS-T/BSA for 1 hour  
          at room temperature. Then the filters were washed another  
30    4 times with TBS-T, followed by one wash with 100 mM Tris,  
          pH 9.0, 100 mM MgCl<sub>2</sub>. Positive clones were identified by a  
          colorimetric assay using 1-Step BCIP/NBT (Pierce).

-28-

### Example 7 mRNA Isolation and Analysis of Identified Clones

mRNA was isolated from tissues and from 3T3-L1 cells at various stages of differentiation. The mRNA was  
5 separated by agarose gel electrophoresis and transferred to nylon membranes and DNA probes were [<sup>32</sup>P] labeled according to (Baldini, G., Holh, T., Lin, H.Y., and Lodish, H.F. (1992) *Proc. Natl. Acad. Sci. USA* 89:5049-5052).  
Hybridization of the labeled probe to the nylon membranes  
10 was performed overnight at 42°C in 50% formamide, 5x SSC, 25 mM Na-phosphate, pH 7.0, 10x Denhardt's solution, 5 mM EDTA, 1% SDS, and 0.1 mg/ml polyA; the [<sup>32</sup>P] DNA probes were used at 2x10<sup>6</sup> cpm/ml. The membranes were subsequently washed in 2x SSC/0.1% SDS and 0.1x SSC/0.1% SDS at 50°C.  
15 The same membranes were thereafter stripped and reprobed with a radiolabeled cDNA encoding one of the constitutively expressed proteins - cytosolic hsp70 (Hunt, C., and Calderwood, S. (1990) *Gene* 87:199-204; Scherer, P.E., Lisanti, M.P., Baldini, G., Sargiacomo, M., Corley-Mastick,  
20 C., and Lodish, H.F. (1994) *J Cell Biol* 127:1233-1243) and a partial clone of mouse Sec23 (Wadhwa, R., Kaul, S.C., Komatsu, Y., Ikawa, Y., Sarai, A., and Sugimoto, Y. (1993) *FEBS Lett* 315:193-196).

Of the clones characterized, five were novel, and five  
25 encoded four known secretory proteins. Two of these were known to be secreted by adipocytes - fibronectin (Spiegelman, B.M., and Ginty, C.A. (1983) *Cell* 35:657-666) and complement factor C3 (Choy, L.N., Rosen, B.S., and Spiegelman, B.M. (1992) *J Biol Chem* 267:12736-12741) -  
30 while others have not been described as adipocyte secretory proteins - osteonectin (Lane, T.F., and Sage, E.H. (1994) *FASEB J* 8:163-173) and alpha-1 acid glycoprotein (Ricca, G.A., Hamilton, R.W., McLean, J.W., Conn, A., Kalinyak, J.E., and Taylor, J.M. (1981) *J Biol Chem* 256:10362-10368).

-29-

One of the clones, B52, was found to be the mouse homologue of the  $\alpha 3$  subunit of human type VI collagen.

The tissue distribution of two of the clones, B52, and osteonectin was tested by Northern blot analysis. B52 was  
5 found to be expressed in fat tissue and 3T3-L1 adipocytes and not in mouse kidney, liver, brain, testis, diaphragm, heart, lung or spleen. Osteonectin was most highly expressed in mouse fat tissue, highly expressed in lung, expressed at a low level in brain, diaphragm, heart and  
10 3T3-L1 adipocytes, but not expressed in kidney, liver, testis and spleen. Expression of the B52 during differentiation of precursor fibroblasts to adipocytes was measured by Northern blot analysis. B52 mRNA was found to be highly induced during differentiation, and can be  
15 detected within two days after the cells reach confluence.

Example 8            Isolation of a full-length  $\alpha 3$ (VI) collagen cDNA

The full-length clone of B52 (9kb) was isolated by a combination of screening the  $\lambda$ EXlox™ cDNA library as well  
20 as a 5' - stretch mouse fat library (Clonetech) and nested PCR. The  $\lambda$ EXlox™ cDNA library and 5' - stretch mouse fat library were screened with digoxigenin-labeled (Boehringer) probes comprising the 300 most 5' nucleotides of the clone obtained by SAS, resulting in overlapping clones that  
25 lacked the 5' most 4000 nucleotides of the putative full-length gene. The 5' end of the full-length gene was obtained by nested PCR using the day 8 adipocyte cDNA library together with vector-based primers for the 5' end and a set of nested primers at the 3' end. PCR products  
30 were subcloned into pZero (Invitrogen) and subjected to automated sequencing.

-30-

**Example 9 Radiolabeling and Immunoprecipitations.**

3T3-L1 adipocytes were incubated for 30 min in DMEM lacking cysteine and methionine (ICN, Costa Mesa) and then labeled for 10 min in the same medium containing 0.5 mCi/ml of <sup>35</sup>S Express Protein Labeling Reagent (1000 Ci/mmol) [NEN (Boston, MA)]. The cells were then washed twice with DMEM supplemented with unlabeled cysteine and methionine and then fresh growth medium was added. After three hours of incubation at 37°C, the medium was collected and the cells lysed in lysis buffer (1% Triton X-100, 60 mM octyl-glucoside, 150 mM NaCl, 20 mM Tris pH 8.0, 2 mM EDTA, 1 mM PMSF). Insoluble material from both the medium and cell lysate was removed by centrifugation (15,000 g for 10 min). The supernatants were precleared with 50 µl Protein A - Sepharose for 30 min at 4°C and then immunoprecipitated with antibody for 3 hrs at 4°C. Immunoprecipitates were washed 4 times in lysis buffer lacking octyl-glucoside.

Separation of proteins by SDS-PAGE, fluorography, immunoblotting, protein determinations and densitometric scanning of the gels were performed as described previously (Scherer et al. 1994). Automated DNA Sequencing was performed on a Applied Biosystems apparatus (Model PE/ABI 373A) by the Whitehead Institute Sequencing Facility.

**EQUIVALENTS**

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:



-31-

## CLAIMS

We claim:

1. A method of identifying a protein that is expressed in a cell type of interest and is not expressed in at least one cell type other than the cell type of interest, comprising the steps of:
  - (a) combining a cDNA expression library and immunodepleted serum, wherein the immunodepleted serum is produced by:
    - i) combining: a) antiserum, produced by immunizing an appropriate host with antigens derived from the cell type of interest; and b) antigens from at least one cell type other than the cell type of interest, under conditions appropriate for binding of antibodies in the antiserum with antigens of b, wherein the antigens bind to antibodies, thereby producing antigen-bound antibodies;
    - ii) separating the antigen-bound antibodies from the antiserum, thereby producing serum, referred to as immunodepleted serum, which does not contain the antigen-bound antibodies,whereby proteins expressed by the cDNA expression library bind antibodies in the immunodepleted serum to form complexes of a protein expressed by the cDNA expression library and an antibody in the immunodepleted serum; and
  - (b) detecting complexes of proteins expressed by the cDNA expression library and antibodies in the immunodepleted serum,

-32-

wherein presence of complexes is indicative of the presence of a protein expressed in the cell type of interest.

2. The method of Claim 1 further comprising isolating a protein that is expressed in a cell type of interest, wherein the complexes of proteins expressed by the cDNA expression library and antibodies are separated from the cDNA expression library and, optionally, wherein the antibody is separated from the protein expressed by the cDNA expression library.
3. The method of Claim 1 further comprising isolating from the cDNA expression library, a gene or fragment thereof encoding a protein expressed in the cell type of interest.
4. The method of Claim 1 wherein the antigens are derived from sub-cellular fractions of the cell type of interest, wherein the sub-cellular fractions are selected from the group consisting of: secreted fractions, plasma membrane fractions, cytoplasmic fractions, organellar fractions, intracellular membrane fractions, nuclear fractions and subfractions of the sub-cellular fractions.
5. The method of Claim 1 wherein the cell type of interest is selected from the group consisting of: adipocytes, transformed cells, malignant tumor cells, C2C12 osteoblasts, C2C12 myotubes, PC12 neurocytes, HNT neurocytes, differentiated blood cells and progenitors thereof.

-33-

6. The method of Claim 1 wherein the antigens that are derived from at least one cell type other than the cell type of interest are modified to allow separation of antibody-antigen complexes from the antiserum by:
  - 5 a) coupling to a solid support selected from the group consisting of: Sepharose, agarose, acrylamide, nitrocellulose, glass, plastic and silicone; or b) chemical addition to the antigens of a functional group which is biotin or digoxigenin.
- 10 7. The method of Claim 1 wherein the cell type of interest is stimulated and the cell type other than the cell type of interest is non-stimulated.
8. The method of Claim 7 further comprising isolating a protein that is expressed in a stimulated cell type of interest, wherein the complexes of proteins expressed
  - 15 by the cDNA expression library and antibodies are separated from the cDNA expression library and, optionally, wherein the antibody is separated from the protein expressed by the cDNA expression library.
- 20 9. The method of Claim 7 further comprising isolating from the cDNA expression library, a gene or fragment thereof encoding a protein expressed in the stimulated cell type of interest.
10. The method of Claim 7 wherein the antigens are derived from sub-cellular fractions of the cell type of interest, wherein the sub-cellular fractions are selected from the group consisting of: secreted fractions, plasma membrane fractions, cytoplasmic fractions, organellar fractions, intracellular
  - 25

-34-

membrane fractions, nuclear fractions and subfractions of the sub-cellular fractions.

11. The method of Claim 7 wherein the stimulated cell type of interest is selected from the group consisting of:  
5 adipocytes, transformed cells, malignant tumor cells, C2C12 osteoblasts, C2C12 myotubes, PC12 neurocytes, HNT neurocytes, differentiated blood cells and progenitors thereof.
12. The method of Claim 7 wherein the cell type of  
10 interest is non-stimulated and the cell type other than the cell type of interest is stimulated.
13. The method of Claim 12 further comprising isolating a  
15 protein that is expressed in a non-stimulated cell type of interest, wherein the complexes of proteins expressed by the cDNA expression library and antibodies are separated from the cDNA expression library and, optionally, wherein the antibody is separated from the protein expressed by the cDNA expression library.
- 20 14. The method of Claim 12 further comprising isolating from the cDNA expression library, a gene or fragment thereof encoding a protein expressed in the non-stimulated cell type of interest.
- 25 15. The method of Claim 12 wherein the antigens are derived from sub-cellular fractions of the cell type of interest, wherein the sub-cellular fractions are selected from the group consisting of: secreted fractions, plasma membrane fractions, cytoplasmic fractions, organellar fractions, intracellular

-35-

membrane fractions, nuclear fractions and subfractions of the sub-cellular fractions.

16. The method of Claim 12 wherein the non-stimulated cell type of interest is selected from the group consisting of: adipocytes, transformed cells, malignant tumor cells, C2C12 osteoblasts, C2C12 myotubes, PC12 neurocytes, HNT neurocytes, differentiated blood cells and progenitors thereof.
17. The method of Claim 1 wherein the cell type of interest is selected from the group consisting of: a transformed cell, a malignant cell and a metastatic malignant cell and the cell type other than the cell type of interest is selected from the group consisting of: a non-transformed cell, a non-malignant cell and a non-metastatic cell.
18. The method of Claim 17 further comprising isolating a protein that is expressed in the cell type of interest, wherein the complexes of proteins expressed by the cDNA expression library and antibodies are separated from the cDNA expression library and, optionally, wherein the antibody is separated from the protein expressed by the cDNA expression library.
19. The method of Claim 17 further comprising isolating from the cDNA expression library, a gene or fragment thereof encoding a protein expressed in the cell type of interest.
20. The method of Claim 17 wherein the antigens are derived from sub-cellular fractions of the cell type of interest, wherein the sub-cellular fractions are

-36-

5 selected from the group consisting of: secreted fractions, plasma membrane fractions, cytoplasmic fractions, organellar fractions, intracellular membrane fractions, nuclear fractions and subfractions of the sub-cellular fractions.

21. The method of Claim 1 wherein the cell type of interest is selected from the group consisting of: a non-transformed cell, a non-malignant cell and a non-metastatic cell and the cell type other than the cell type of interest is selected from the group consisting of: a transformed cell, a malignant cell and a metastatic malignant cell.
- 10
22. The method of Claim 21 further comprising isolating a protein that is expressed in the cell type of interest wherein the complexes of proteins expressed by the cDNA expression library and antibodies are separated from the cDNA expression library and, optionally, wherein the antibody is separated from the protein expressed by the cDNA expression library.
- 15
- 20 23. The method of Claim 21 further comprising isolating from the cDNA expression library, a gene or fragment thereof encoding a protein expressed in non-transformed cells.
24. The method of Claim 21 wherein the antigens are derived from sub-cellular fractions of the cell type of interest, wherein the sub-cellular fractions are selected from the group consisting of: secreted fractions, plasma membrane fractions, cytoplasmic fractions, organellar fractions, intracellular
- 25

-37-

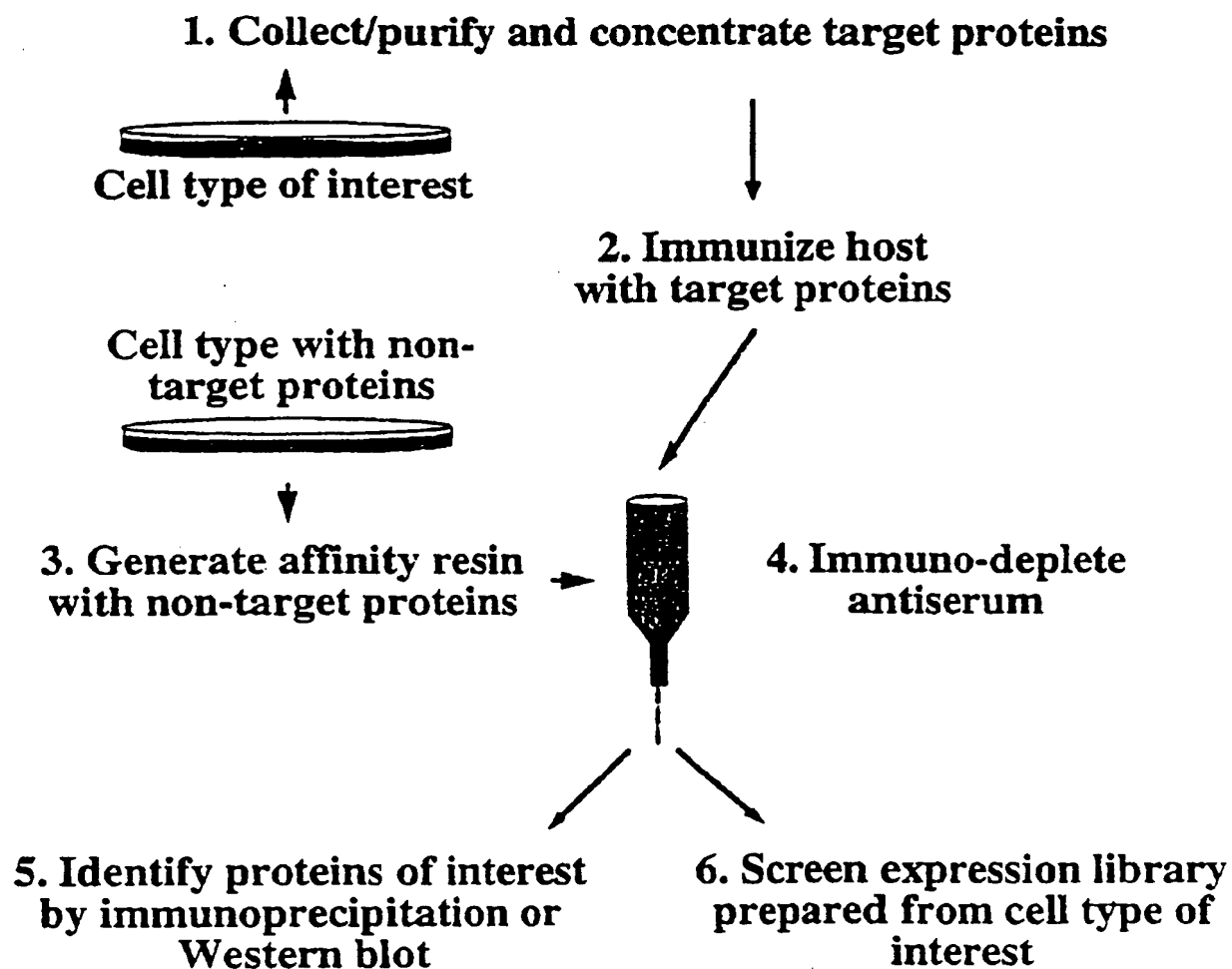
membrane fractions, nuclear fractions and subfractions of the sub-cellular fractions.

25. A method of identifying a protein that is expressed in adipocytes and not expressed in fibroblasts,  
5 comprising the steps of:
- (a) combining a cDNA expression library of adipocytes and immunodepleted serum, wherein the immunodepleted serum is produced by:
    - 10 i) combining: a) antiserum, produced by immunizing an appropriate host with antigens derived from adipocytes; and b) antigens from fibroblasts, under conditions appropriate for binding of antibodies in the antiserum with antigens of b), wherein the  
15 antigens bind to antibodies, thereby producing antigen-bound antibodies;
    - ii) separating the antigen-bound antibodies from the antiserum, thereby producing serum, referred to as immunodepleted serum, which  
20 does not contain the antigen-bound antibodies,
- whereby proteins expressed by the cDNA expression library bind antibodies in the immunodepleted serum to form complexes of a protein expressed by  
25 the cDNA expression library and an antibody in the immunodepleted serum; and
- (b) detecting complexes of proteins expressed by the cDNA expression library and antibodies in the immunodepleted serum, wherein presence of  
30 complexes is indicative of the presence of a protein expressed in adipocytes.

-38-

26. The method of Claim 25 further comprising isolating a protein that is expressed in adipocytes, wherein the complexes of proteins expressed by the cDNA expression library and antibodies are separated from the cDNA expression library and, optionally, wherein the antibody is separated from the protein expressed by the cDNA expression library.
27. The method of Claim 25 further comprising isolating from the cDNA expression library, a gene or fragment thereof encoding a protein expressed in adipocytes.
28. The method of Claim 25 wherein the antigens are derived from sub-cellular fractions of the cell type of interest, wherein the sub-cellular fractions are selected from the group consisting of: secreted fractions, plasma membrane fractions, cytoplasmic fractions, organelar fractions, intracellular membrane fractions, nuclear fractions and subfractions of the sub-cellular fractions.
29. The method of Claim 25 wherein the antigens that are derived from fibroblasts are modified to allow separation of antibody-antigen complexes from the antiserum by: a) coupling to a solid support selected from the group consisting of: Sepharose, agarose, acrylamide, nitrocellulose, glass, plastic and silicone; or b) chemical addition to the antigens of a functional group which is biotin or digoxigenin.





FIGURE

# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/US 98/00588

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 G01N33/68 G01N33/569 G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BATRA, S.K. ET AL.: "A simple, effective method for the construction of substracted cDNA libraries" GENET. ANAL.: TECH. APPL, vol. 8, no. 4, 1991, pages 129-133, XP002066340 see the whole document -----	1-29
A	WILSON, B.E. ET AL.: "Identification of candidate genes for a factor regulating body weight in primates." AM. J. PHYSIOL., vol. 259, no. 6/2, 1990, pages R1148-R1155, XP002066341 see the whole document -----	1-29

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

28 May 1998

Date of mailing of the international search report

16/06/1998

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Hoekstra, S